KAVAKA 41: 68-74 (2013)

Studies on destruxin production and toxicity by entomopathogenic fungus *Metarhizium anisopliae* ARSEF-2735

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(Received on 23 November 2013; Accepted on 31 December 2013)

ABSTRACT

The entomopathogenic fungus *Metarhizium anisopliae* is known to produce destruxins which are low molecular weight secondary metabolite. The great interest in destruxins as study model results from their potential as virulence factors for insect pests. Various physical and biological factors influence the rate of destruxin production. The present study deals with studies on characterisation and destruxin production by *Metarhizium anisopliae* ARSEF-2735, and variation in destruxin production, its pH and biomass with respect to different media and we report that Carbon and Nitrogen sources have a major role to play in the destruxin production and colloidal chitin (1 %) is a positive elicitor for its production. Bioassays were designed to evaluate the virulence potential of crude destruxins on banded winged whiteflies and it is observed to possess commendable anti-feedant properties. The highest insect mortality was observed with - Czapek Dox (CZ) supplemented with 1 % colloidal chitin (LD_{50} = 16.82), followed by CZ with 1 % cockroach homogenate (LD_{50} = 29.50), and least for basal CZ media (LD_{50} = 67.87). The studies further confirms the importance of colloidal chitin as the necessary inducer for fermentative production of destruxin using *M. anisopliae*.

Keywords: *Metarhizium anisopliae,* destruxins, bioassays, whitefly, toxicity

INTRODUCTION

The entomopathogenic fungus *Metarhizium anisopliae* is cosmopolitan and infects a wide range of host insects. It is known to produce low molecular weight, bioactive secondary metabolite, destruxin, which is a cyclodepsipeptides. These are of special interest in context of the biological control of insect pests as these are the only mycotoxins detected in the insect body at advanced stages of infection in sufficient quantities to cause mycosis (Brousseau et al. 1996; Kershaw et al. 1999). In the group of destruxins biosynthesized by *M. anisopliae* isolates, the destruxin A, B and E are predominant. Destruxin E is the most toxic for invertebrates, particularly insects (Dumas et al. 1994; Loutelier et al. 1996; Pedras et al. 2002; Strasser et al. 2000). Biochemical and analytical studies showed that both *in vivo* and *in vitro* diffusion of destruxin is a very rapid process from endogenous mycelia, although their ratios being different (Cherton et al. 1991; Amiri-Besheli et al. 2000; Butt et al. 1994). Bio-pharmacological studies reveal that the paralysis induced by destruxin in insects results from a calcium-dependent effect on muscular cells (Kodaira, 1961; Samuels et al. 1988a, b, c). It is suggested that destruxins also inhibit Malphigian tubule fluid secretion (James et al. 1993) as well as ecdysteroid secretion by prothoracic glands (Sloman and Reynolds, 1993). Several studies have reported immunomodulatory effects of destruxins (Huxam et al. 1989; Vilcinskas et al. 1997) and reveal that inhibition of cellular immune response in the haemolymph may be the key factor for destruxin induced mycosis. It is established that destruxin induce calcium influx and phosphorylation of intracellular proteins within lepidopteron cell lines (Dumas et al. 1996). A positive correlation between virulence and destruxin production has been evidenced from several studies.

The destruxin production is not only influenced by biological factors, the physicochemical environment in which the organism is grown also plays an important role in the high yield production. In particular, pH and aeration rate are the most critical parameters and plays a significant role in productivity of the process (Bing-Lan Lin et al. 2007). It is reported that destruxin production is highly influenced by the component type and the C:N ratio of the culture media (Liu et al. 2000; Wang et al. 2004).There are considerable evidences that suggest substantial involvement of destruxin in pathogenesis by *M. anisopliae*. The current chemical research, flexibly allow preparation of semi-synthetic analogues which opens

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avenues for the development of comparative research (Hsiao and Ko, 2001). In this study, destruxin production by *M. anisopliae* isolate (ARSEF 2735) was monitored in different culture and induction media. The relationship between the destruxin production, pH and biomass variation were analysed and discussed. The destruxin was also assessed for its biocidal effects on the adults of banded winged whiteflies *Trialeurodes abutilonea*.

MATERIALS AND METHODS

Fungal Culture

The isolate UM2 (ARSEF 2735), was routinely subcultured on Sabouraud Dextrose Agar (SDA) at 28°C in the dark and the slants were kept at 4^0C when completely sporulated, usually after 10 days. For longer term storage, conidial suspensions were prepared in 20% glycerol solution and stored at -20^0C. These stocks were used as inoculum for fresh culture.

Culture media

The impact of different nutrient supplements on the level of destruxin production was evaluated by growing the fungal culture in six different media. The Czapek Dox (CD) medium and the Minimal Medium (MM, CD without sucrose and peptone) were the two basic media used for the study. Further, we used Colloidal Chitin (CC, 1%) and Cockroach Homogenate (CH, 1%) supplements (CD+CC, CD+CH, MM+CC and MM+CH) to see if at all it has some induction effect on destruxin production. Colloidal chitin was prepared with a diminutive modification of the Simhara and Takiguchi (1988) method. For preparation of cockroach homogenate, the adult cockroach *Periplanata americana*, were frozen to death for 45min at -80^0C and homogenized under liquid nitrogen. The homogenates were then oven-dried at 60^0C for 48h. Supplementing the CD and MM with cockroach homogenate and colloidal chitin were included in the study to compare the induction effect of chitin on toxin production (Wang et al. 2004). The pH of all the media were adjusted to 7.0 using 1M NaOH. Conidia were harvested from sporulating plates using 0.02% Tween 80 solution and the suspension was agitated for 45s to reduce clumping. The concentration of conidia was determined by use of a haemocytometer. The viability of spores was assessed by spreading them onto a glass slide coated with SDA and incubated for 24 h at 28^0C in the dark. Around 600x 10^6 conidia were inoculated in each of the test media. The control flasks were not inoculated. The culture filtrates were harvested by centrifugation after incubation, every 5th day for 30 days. The destruxins were extracted with dichloromethane. The destruxin production was confirmed by Liquid Chromatography Mass Spectrometric analysis (Fig 1). The destruxin was lyophilized and was used in various dilutions for the bioassays.

Insect bioassays

The banded winged whitefly *Trialeurodes abutilonea* (Homoptera: Aleyrodidae) adult flies were used for determination of LD_{50}. The insects were initially fed on the flowers and foliage of *Ixora coccinia* flowers and foliage and were maintained at 25^0C-28^0C.

For bioassays, destruxins produced from the three media (CD, CD+CC and CD+CH) was used. The crude destruxin powder obtained was initially dissolved in ethyl acetate to prepare stock solutions of crude destruxin by sufficient vortexing to dissolve the crude powder completely. The stock from each source was diluted 10, 100 and 1000 times to prepare five working concentrations (0X, 1X, 10X, 100X and 1000X; 0X being the control setup where only solvent

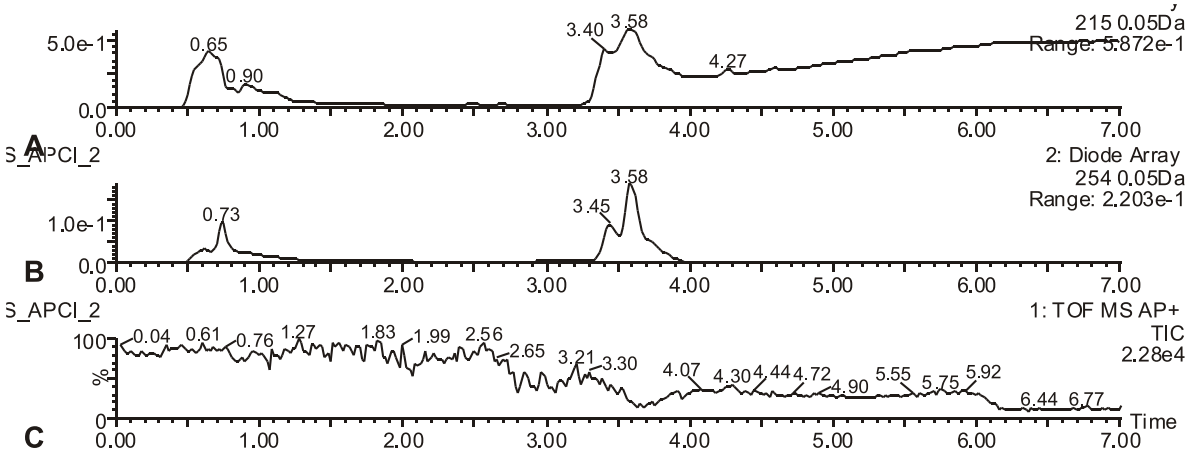

Fig. 1. Liquid Chromatography Mass Spectrometry (LCMS) based chromatogram of destruxin analysis as observed in APCI positive ion mode. A. The chromatogram obtained at 215nm. B. The chromatogram obtained at 254nm. C. The Total Ion Count. The peaks upon analysis showed presence of destruxin A, Desm A, B, C_2, D, Ed, E_1 and Desm D_1.

was present) for each of the treatments (Table 1). The various diluents of the crude destruxin were spread evenly on the *Ixora coccinia* leaves and were allowed to dry in the laminar hood for some time to allow the solvent to evaporate. The insects being sap suckers were solely fed with the crude destruxins treated *Ixora coccinia* leaves and maintained at 12h light- 12h dark condition at room temperature (25^0C-28^0C) and were observed every 10h for any mortality.

Statistical analysis

Statistical analysis of all the data for destruxin concentration, pH and biomass variation were subjected to one-way analysis of variance (ANOVA) and the means were separated by Student-Newman-Keuls multiple range test of comparisons of means at P= 0.05. The mortality data was subjected to Probit Analysis (Finney, 1964) for calculating the LD_{50} values using the software BIOSTAT 2009, Version 5.7.4.0. Correlations between the production of destruxin as well as pH and biomass were analyzed by calculating Pearson's correlation coefficient with the program SPSS 17.0.0 (SPSS Inc.).

RESULTS

pH, Biomass and crude destruxin production

The destruxin production was tested by LCMS (Fig.1). The pH of all the media (except MM) studied for destruxin production kept decreasing from day 5 - 30 post inoculation (Table). It was observed that the pH which was initially fixed at 7.0 at the start of the experiment was gradually lowered to acidic range. The decrease in pH was more pronounced in CD + CC and CD alone, where it reduced to 3.41 and 3.42 respectively after 30 dpi. The fungal biomass kept increasing up to day 15 in all the cases, after which it remained almost constant for all the media (Table 2). The biomass production was least in MM supplemented with CC and CH (0.97 mg/ 100 ml and 0.89 mg/ 100 ml); while in MM alone, the biomass was nil with negligible destruxin production. All the substrates studied, showed an increase in destruxin production from day 5 to day 10 post inoculations, and thereafter a gradual decline in the destruxin production rate (Table 2). Maximum destruxin production of about 16.39 mg/ 100 ml was observed in the CD media supplemented with colloidal chitin (1 %), followed by CD media supplemented with cockroach homogenate (1 %) with concentration of 13.45 mg/ 100 ml, followed by CD as the sole nutrient substrate. The MM media supplemented with CC (1 %) and CH (1 %) showed a meager rate of destruxin production while the Minimal Media as the sole nutrient source produced negligible biomass and almost no toxin. The pH and biomass were significantly associated (at á = 0.01 for CD + CC (1 %), and at á = 0.05 for the rest of the substrates), but no significant association was observed between toxin production and variation in pH and biomass (Table 3).

Bioactivity of destruxins

For insect bioassays, destruxins produced from the three media (CD, CD+CC and CD+CH) were used. All the three media produced varying amount of crude destruxin (Table 2) and 10 day old cultures of each was used for estimation of LD_{50} values. The media CD+CC produced high amount of crude destruxin (16.39 mg) followed by media CD+CH (13.45 mg) and CD (9.99 mg). The bioassay procedures for each of the destruxin sample from different media were same but the observations of each media were analyzed separately. Virulence was expressed as LD_{50} which is defined as the statistically derived exposure dosage of a pesticide expected to cause mortality in 50 % of an insect population. The LD_{50} value of CD+CC media at 30 h post treatment was the lowest (16.82) as the initial concentration of crude destruxin was also high followed by CD+CH (29.50) and CD (67.87) respectively. In all the three media

Table 1: The details of the crude destruxins and its serial dilutions for insect bioassays#

Media*	Extracting solvent (100 ml each)	Quantity of crude destruxin (mg)**	Stock ($mgml^{-1}$)***	Serial dilutions for Bioassays****				
				0X	1X	10X	100X	1000X
CD	DCM	9.99	Dissolving entire quantity in 1 ml of EA	Solvent EA only	9.99	0.999	0.0999	0.00999
CD + CC	-do-	16.39	-do-	-do-	16.39	1.639	0.1639	0.01639
CD + CH	-do-	13.45	-do-	-do-	13.45	1.345	0.1345	0.01345

DCM: Dichloromethane.
EA: Ethyl Acetate.
#The bioassay procedures for each of the destruxin sample from different media were same but the observations of each media were analyzed separately.
*Ten day old cultures from different media were used
**Quantity represents the amount of crude destruxin obtained in mg from 50 ml of the inoculated culture per 10^6 conidia concentration as the starting material. These were the values obtained when Dichloromethane was used as the extracting solvent.
***Stock was prepared by re-dissolving (in Ethyl acetate) the total amount of the crude destruxin obtained after evaporation of the extracting - solvent (Dichloromethane).
****The serial diluents are in $mgml^{-1}$, the same as that of stock solution.

Table 2: Variation in pH, Biomass and destruxin production on different media

	Days	Media					
		CD	**CD +CC**	**CD + CH**	**MM**	**MM + CC**	**MM + CH**
pH	5	6.34[a]	6.43[a]	6.13[a]	Negligible variation in pH	6.34[a]	6.11[a]
	10	5.85[b]	5.52[b]	5.41[b]		5.85[b]	5.83[b]
	15	5.17[c]	4.79[c]	5.11[b]		5.78[b]	5.60[c]
	20	4.25[d]	4.17[d]	4.71[c]		5.31[c]	5.17[d]
	25	3.43[e]	3.67[e]	4.64[c]		5.13[d]	4.94[e]
	30	3.42[e]	3.41[f]	3.65[d]		4.75[e]	4.60[f]
		Media					
	Days	**CD**	**CD +CC**	**CD + CH**	**MM**	**MM + CC**	**MM + CH**
Biomass	5	0.45[b]	0.65[c]	0.25[c]	Almost nil	0.21[c]	0.25[b]
	10	0.54[b]	0.95[c]	0.73[b]		0.63[b]	0.44[b]
	15	1.24[a]	1.39[b]	1.46[a]		0.91[a]	0.87[a]
	20	1.37[a]	1.89[a]	1.70[a]		0.97[a]	0.93[a]
	25	1.36[a]	1.83[a]	1.80[a]		0.92[a]	0.87[a]
	30	1.31[a]	1.90[a]	1.68[a]		0.97[a]	0.89[a]
		Media					
Destruxin production	**Days**	**CD**	**CD +CC**	**CD + CH**	**MM**	**MM + CC**	**MM + CH**
	5	5.06[fC]	8.26[fA]	6.54[eB]	Almost nil	0.18[dD]	0.18[dD]
	10	9.99[aC]	16.39[aA]	13.45[aB]		0.84[aE]	1.03[aD]
	15	8.78[bC]	15.12[bA]	12.30[bB]		0.79[aD]	0.67[bE]
	20	7.54[cC]	13.60[cA]	10.95[cB]		0.45[bE]	0.65[bD]
	25	7.38[dC]	13.03[dA]	11.03[cB]		0.25[cD]	0.36[cD]
	30	6.75[eC]	12.28[eA]	10.09[dB]		0.07[eD]	0.09[eD]

Values followed by the same lower case alphabets in the same column are statistically equivalent ($P < 0.05$) according to the Newman-Keul's multiple range test.
*The amount of crude destruxin powder (in mg) obtained from 50 ml of the inoculated culture per 10^6 conidia concentration as the starting material.

it was observed that greater the dilution lesser the mortality caused as it was nil with the 1000x dilution. The other dilutions too showed the same trend where as the concentration of crude destruxin decreased, the mortality also decreased respectively. The LD_{50} values at 60 hr of observation, for each media was found to be lower than that at 30 hr of observation thus implying that lesser dose of the toxin required with increasing time of treatment (Table 4). This was true for all the media studied. More the destruxin concentration, lesser the lethal dose required for the desired kill rate and as the concentrations were different for all the three media studied the LD_{50} values also varied respectively.

DISCUSSION

Destruxin have been implicated as one of the causes of insect death infected with *M. anisopliae* (Vey et al. 2001) and they are the only reported toxins from the mycosed insect hosts (Amiri-Besheli et al. 2000). Wang et al. (2009) reported that destruxin production is highly influenced by nitrogen availability. Carbon and Nitrogen sources have a major role to play in the destruxin production because when devoid of the

Table 3: Pearson relationships between variations in pH, biomass and destruxin concentration as observed during a period of 30 days

Media	Parameters	pH	Biomass	Destruxin concentration
CD	pH	1	-0.883*	0.051
	Biomass	-0.883*	1	0.061
	Destruxin concentration	0.051	0.061	1
CD + CC	pH	1	-0.973**	-0.286
	Biomass	-0.973**	1	0.258
	Destruxin concentration	-0.286	0.258	1
CD + CH	pH	1	-0.843*	-0.280
	Biomass	-0.843*	1	0.414
	Destruxin concentration	-0.280	0.414	1
MM + CC	pH	1	-0.838*	0.397
	Biomass	-0.838*	1	0.078
	Destruxin concentration	0.397	0.078	1
MM + CH	pH	1	-0.824*	0.389
	Biomass	-0.824*	1	-0.063
	Destruxin concentration	0.389	-0.063	1

* Correlation is significant at the 0.05 level (2 - tailed).
** Correlation is significant at the 0.01 level (2 - tailed).

Table 4: Mortality data and Probit Analysis (Finney Method [Lognormal Distribution]) to estimate LD_{50}* values on different media

Media	Serial dilution for bioassays*	Mortality (%) at 30 h	Mortality (%) at 60 h	LD_{50}[a]	LD_{50}[b]
CD	0X	0	0	67.87	0.96
	1X	30	80		
	10X	20	40		
	100X	10	30		
	1000X	0	10		
CD+CC	0X	0	0	16.82	0.61
	1X	50	10		
	10X	20	50		
	100X	10	30		
	1000X	0	10		
CD+CH	0X	0	0	29.50	0.68
	1X	40	90		
	10X	20	50		
	100X	10	30		
	1000X	0	10		

*Dilutions as specified in Table 2

[a]The values were recorded 30 hrs post treatment.

[b]The values were recorded 60 hrs post treatment.

same, the fungus does not support appreciable destruxin production.

Wang et al. 2004, reported negative correlation of destruxin A and B on pH variation of the culture media, but found no significant association between pH and destruxin E production. Studies also suggest addition of supplements like carbon and nitrogen sources or cyclopeptolide (Espada and Dreyfuss, 1979) or use of antioxidants (Rao et al. 2006) as destruxin production enhancers. In our study, when Sucrose (component of CD media) was used as the main Carbon source (CD, CD + CC, CD + CH), the biomass and destruxin production was more than in the absence of it (MM, MM + CC, MM + CH). Similarly, when peptone (component of CD media) was used as the main nitrogen source (CD, CD + CC, CD + CH), the biomass and destruxin production was more than in the absence of it (MM, MM + CC, MM + CH). When the two basic media (CD and MM) were supplemented with CC and CH (as in CD + CC, CD + CH, MM + CC AND MM + CH), the destruxin production as well as the biomass produced was more in the non-supplemented media. Further it was observed that destruxin production was more in the CD + CC than in CD + CH, and in the MM series, it was observed to be more in MM + CC than in MM + CH, thereby indicating that pure chitin i.e. Colloidal Chitin (CC) obtained commercially from Sigma, was a better elicitor of destruxin production than its crude counterpart i.e. Cockroach Homogenate (CH) derived chitin. We can also say that the same amount of colloidal chitin from Sigma, contributes more chitin to the media than the crude chitin obtained by homogenization of cockroach; thus we can safely conclude that more the amount of chitin, higher is the destruxin elicitation. Wang et al. (2004) on the other hand did not observe any toxin production when using insect homogenate as single nutrient or when included in the liquid culture media.

The bioactivity of destruxin to various insects has already been reported, having different mode of actions such as contact, antifeedant and growth regulation (Hu et al. 2009). Kershaw et al. (1999), advocated two major modes of myco-pesticidal effect to insects, viz., the 'toxin strategy' in which the fungus has limited growth in the haemolymph and produces destruxin in sufficient quantity to have a direct involvement in the cause of insect mortality; and secondly, the 'growth strategy' which basically involves mechanical pressure and results in the profuse growth of the fungus over and within the insect, thereby leading to homeostasis disruption and starvation causing insect death. In this study, we used the crude destruxin dilutions (sans the fungal spores and mycelium from which it was extracted) and therefore the bioassay strategy followed here was 'toxin – strategy'.

The present study shows that the Lethal Doses to ascertain the insect mortality (here whiteflies) have an inverse relationship with the time of treatment. It was observed that under similar conditions of bioassays, (like same type serial diluents, same number of insects, same bioassay procedures) more the initial concentration of the destruxin, lesser was the Lethal Dose to kill the insects. Another major finding was that the crude destruxins have commendable anti-feedant activity. The toxin-based bioassays are unlike the majority of bioassays which are conidia-based. It is also different from other toxin-based bioassays, where usually only one type of commercially available toxin is used in various dilutions; maybe that is to find the contribution of individual destruxin member towards pathogenesis but we were more interested to prefer crude destruxin extract (which was a mixture of a number of destruxins) to see the effect of toxin – strategy on host insect, because in natural environment the destruxin family acts synergistically to cause entomopathogenesis.

ACKNOWLEDGMENTS

We acknowledge the financial support provided by the Department of Science and Technology (Project No. SR/FT/L-144). We also thank Dr. Richard A. Humber (USDA-ARS) for providing the fungal cultures.

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